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Induction of Acquired Thermotolerance in *Tetrahymena thermophila*: Effects of Protein Synthesis Inhibitors

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When *Tetrahymena thermophila* cells growing at 30°C are shifted to either 40 or 43°C, the kinetics and extent of induction of heat shock mRNAs in both cases are virtually indistinguishable. However, the cells shifted to 40°C show a typical induction of heat shock protein (HSP) synthesis and survive indefinitely (100% after 24 h), whereas those at 43°C show an abortive synthesis of HSPs and die (<0.01% survivors) within 1 h. Cells treated at 30°C with the drugs cycloheximide or emetine, at concentrations which are initially inhibitory to protein synthesis and cell growth but from which cells can eventually recover and resume growth, are after this recovery able to survive a direct shift from 30 to 43°C (ca. 70% survival after 1 h). This induction of thermotolerance by these drugs is as efficient in providing thermoprotection to cells as is a prior sublethal heat treatment which elicits the synthesis of HSPs. However, during the period when drug-treated cells recover their protein synthesis ability and simultaneously acquire the ability to subsequently survive a shift to 43°C, none of the major HSPs are synthesized. The ability to survive a 1-h, 43°C heat treatment, therefore, does not absolutely require the prior synthesis of HSPs. But, as extended survival at 43°C depends absolutely on the ability of cells to continually synthesize HSPs, it appears that a prior heat shock as well as the recovery from protein synthesis inhibition elicits a change in the protein synthetic machinery which allows the translation of HSP mRNAs at what would otherwise be a nonpermissive temperature for protein synthesis.

Evidence that one or more of the proteins synthesized in response to a sublethal heat shock (18, 22, 24, 26) or other stressful conditions (21, 29) play some mechanistic role in allowing cells to subsequently survive at temperatures which would otherwise be lethal is compelling but circumstantial. Reports indicate that not all of the heat shock proteins (HSPs) need to be involved in this protection (4, 7, 23), and evidence has also been presented indicating that no HSP synthesis is necessary for the induction of this acquired thermotolerance (13). Whichever the case, the changes brought about in cells which permit survival at these otherwise lethal temperatures are unknown.

One cellular function shown to be altered by heat shock in a variety of different cell types is translation (2, 3, 5, 17, 19, 28, 32, 33). We therefore sought to determine whether a heat shock-induced change in the translational properties of the cell plays any role in permitting cells to subsequently survive at normally lethal temperatures. We examined the induction and utilization of HSP mRNAs in *Tetrahymena thermophila* cells transferred to a nonlethal, heat shock-inducing temperature (40°C) (10) and compared the results to those found in a similar experiment in which cells were transferred to a temperature (43°C) which was lethal but to which cells could be adapted by a prior heat shock. The results of these experiments strongly suggested that translational regulation was at least in part involved in the ability of cells to survive at 43°C. Earlier work has indicated that ribosomes are modified during heat shock (9, 31) and altered in their translational properties (32). We therefore treated cells in ways we knew from previous studies (14, 16) elicited changes in ribosome structure and function to determine whether such treatments had any effect on the ability of cells to survive a direct transfer from 30 to 43°C. We found conditions which induced acquired thermotolerance to 43°C as efficiently as a prior heat shock, but, surprisingly, unlike previous findings for other organisms (21, 29), HSP synthesis was not detectable during the induction of the acquired-thermotolerance state. The conditions which induced this acquired thermotolerance to 43°C, however, did not induce acquired thermotolerance to a 46°C treatment, a condition which can be induced by a prior heat shock. The results of our studies indicate that there are normally lethal temperatures to which *T. thermophila* can be pre-adapted with treatments that allow cells to efficiently translate heat shock mRNAs at these temperatures and thus survive. These treatments need not elicit the synthesis of any of the major HSPs prior to the shift to the lethal temperature. There are other (higher) lethal temperatures which can be withstood if and only if the accumulation of HSPs has occurred prior to the shift to these temperatures.

MATERIALS AND METHODS

Culture conditions. For all experiments, except where noted, we used a single strain of *T. thermophila*, CU355 (IV). We routinely grew cells at 30°C on a gyratory shaker in 1% proteose peptone (Difco Laboratories)–0.003% Sequestrene (CIBA-GEIGY Corp.). We considered cells to be in the early log phase of cell growth only if they were at a concentration of <100,000/ml and doubling every 2.75 h or less. Heat shocks were administered to cells by transferring culture flasks from a 30°C incubator to either a 40, 43, or 46°C shaking water bath. We used volumes of cell suspensions such that the temperature shift was complete within 3 min of the transfer.

Cell viability measurements. The effect of heat shock on the viability of cells was determined as follows. Cell cultures at 30°C were transferred to either 43 or 46°C. At various intervals after the temperature shift, cell samples were removed and transferred to 30°C. Cells were allowed to recover for 2 h, after which the fraction of normal, swimming

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cells was determined. This was accomplished by diluting the cell suspensions to a point at which they could be examined in a hemocytometer and an accurate count of swimming cells could be made. Corrections were made for the dilution, and the number of swimming cells per milliliter of the original culture was calculated. Between 2 and 3 h after the cultures were transferred back to 30°C, 100 to 200 individual cells were collected and transferred into wells of microtiter plates containing growth medium. These were incubated overnight at 30°C. The percentage of cells which could divide and give rise to colonies was determined by visual inspection. In all cases, >90% of the swimming cells collected after 2 h were capable of dividing. No nonswimming cells (most dead cells had lysed by this time) collected 2 h after the return to 30°C ever showed a subsequent revival. As surviving cells do not begin dividing again until 3 or 4 h after being heat shocked at 43 or 46°C, the number of swimming cells counted at 2 h was not affected by cell fission.

Labeling of cell proteins. To label the cell proteins we used [^{3}H]lysine ([4,5-^{3}H]lysine, 70 to 90 Ci/mmol; Amersham Corp.) as a precursor. This amino acid was chosen because its concentration in proteose peptone is extremely low. Thus, there was little reduction of the specific activity of the labeled lysine. For the fluorographic analysis of cell protein, we typically labeled 10^6 cells in 3 to 6 ml of medium containing 10 to 20 μCi of [^{3}H]lysine per ml. Labeling periods ranged from 10 to 30 min. At the end of the labeling the cells were collected and processed as described below.

To determine the rate of incorporation of amino acids at times during heat shock, we pulse-labeled cells for 10 to 20 min with [^{3}H]lysine (20 μCi/ml) at various intervals. At the end of a pulse, three 100-μl samples were removed, and each was mixed with 100 μl of 0.5 M NaOH to lyse the cells. Cold 10% trichloroacetic acid (3 ml) was then added to precipitate the proteins, which were subsequently collected and washed on glass fiber filters. Filters were counted in a liquid scintillation counter, and the average value for each time point was determined. Zero time background incorporation was subtracted for each time point.

Protein electrophoresis techniques. Total cell proteins were prepared for electrophoretic analysis essentially by the method of Guttman et al. (12). A volume of a particular cell suspension containing approximately 10^6 cells was centrifuged to pellet the cells, which were then resuspended in 1 ml of 10 mM Tris (pH 7.5). The cells were repelleted, the washing solution was aspirated off, and 100 μl of lysis buffer (0.05 M Tris [pH 6.8], 1.5% sodium dodecyl sulfate [SDS], 7.5% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin) was added. After mechanical disruption of the cell pellet by vortexing, the lysate was heated to 100°C for 3 min. If not immediately used for electrophoresis, samples were stored at −70°C.

One-dimensional gel electrophoresis was performed by the method of Guttman et al. (12). SDS-containing 15 to 20% polyacrylamide slab gels were used in all experiments. After the stacking gel had been polymerized, the gel was cooled to 4°C for 1 h and then prerun at 10 mA per gel for an additional 1 h. Samples of the cell lysates (10 μl per lane) were electrophoresed at 4°C for 8 h at 20 V/cm. Subsequently, gels were stained with 0.05% Coomassie brilliant blue R in 50% methanol–10% acetic acid, destained in 10% methanol–10% acetic acid, and fluorographed (20).

Two-dimensional gel electrophoresis was also carried out by the method of Guttman et al. (12). The mixture of Ampholines (LKB Instruments, Inc.) used for the one-dimensional gel consisted of equal parts of the following pH ranges: 3.5 to 10, 4 to 6, 5 to 7, and 7 to 9. To 65-μl portions of each of the protein samples prepared as described above were added (in the following order) 3.5 μl of the Ampholine mixture, 50 mg of purified urea, and 10 μl of Nonidet P-40 (Tridom Chemical Co.). The entire mixture was layered onto a 4% polyacrylamide tube gel (3 mm diameter by 11 cm long), overlaid with 20 μl of 8 M urea–6% Nonidet P-40–2% Ampholines and then electrophoresed for 2,500 V-h toward the anode. The gel was then removed from the tube and equilibrated for 1 h in 5 ml of 0.0625 M Tris (pH 6.8)–10% glycerol–5% 2-mercaptoethanol–2.3% SDS. The gel was then transferred to a 7 to 17.5% SDS-polyacrylamide slab gel with a 4% polyacrylamide stacking gel and run at 100 V for 5 to 7 h. The resulting gel was stained, destained, and fluorographed as described above for the one-dimensional gel.

Polysome preparations. To isolate polysomes, approximately 2×10^7 cells were collected by centrifugation and then disrupted in 3 ml of polysome lysis buffer (30 mM Tris, 20 mM potassium acetate, 30 mM $MgCl_2$, 2% spermidine, heparin [1 mg/ml], 2 mM dithiothreitol, cycloheximide [2 μg/ml], 0.5% Nonidet P-40; pH 7.0). The lysate was spun at 10,000 rpm for 10 min, and the resultant supernatant was then divided into two aliquots. One aliquot was brought to 3 ml with an equal volume of lysis buffer. The other aliquot was treated with an equal volume of a solution (0.05 M Tris, 0.2 M KCl, 0.01 M EDTA; pH 7.0) which disrupts polysomes (6). The treated and untreated supernatants were layered over 3-ml 50% sucrose pads (untreated: the sucrose contained 25 mM Tris, 10 mM potassium acetate, 25 mM $MgCl_2$, and 1.0 μg of cycloheximide per ml [pH 7.4]; EDTA-treated: the sucrose contained 25 mM Tris, 0.1 M KCl, and 10 mM EDTA [pH 7.4]) and centrifuged for 1 h at 39,000 rpm and 4°C in a Beckman 50 Ti rotor. After centrifugation, supernatants and pellets were separated, and the RNA was extracted as described below. The validity of this procedure for separating polysomal RNA from nonpolysomal RNA was established by showing that in lysates of long-starved cells 5 to 10% of the rRNA is associated with polysomes, whereas in lysates of exponentially growing cells 90% of the rRNA is associated with polysomes under these conditions. In each case 90% of the material which would have normally pelleted is EDTA releasable to a nonpelletable form under the centrifugation conditions used.

The behavior of rRNAs under these conditions agrees with the data of Calzone et al. (6) who, using other techniques, showed that 5% of the ribosomes in long-starved cells and 90% of the ribosomes in growing cells are associated with polysomes.

RNA isolation. (i) Total cellular RNA. All solutions coming into contact with RNA were autoclaved, and all glassware was heat treated at 165°C for at least 4 h. Cells (10^6 to 10^7) were collected by centrifugation and lysed with 2 to 5 ml of RNA lysis buffer (0.1 M NaCl, 10 mM EDTA, 10 mM Tris hydrochloride [pH 7.4], 1% SDS, 0.5 mg of heparin per ml). After 5 to 10 min in lysis buffer at room temperature, protein was removed by phenol-chloroform-isoamyl alcohol (25:24:1) extraction followed by two chloroform-isoamyl alcohol (24:1) extractions. The RNA was precipitated by adding a 0.1 volume of 3 M sodium acetate (pH 7) and 2.5 volumes of 95% ethanol. After centrifugation at 12,000 × *g* for 15 min, the pellet was washed with ice-cold 70% ethanol, dried, and dissolved in distilled water or TE buffer (10 mM Tris hydrochloride [pH 8], 1 mM EDTA).

(ii) Polysomal RNA. The RNA in the supernatants from a

polysome isolation or in the pellets, which were resuspended in polysome lysis buffer, was extracted exactly as described above for total cellular RNA.

RNA gel electrophoresis. (i) Northern transfers. RNA denatured with glyoxal by the method of McMaster and Carmichael (25) was run on 1% agarose gels as previously described (15).

(ii) Visualization of RNA. RNA was run on 1.8% agarose gels, stained with ethidium bromide, and photographed as previously described (15).

Gel-to-filter transfers and slot blots. Stained RNA gels were washed in distilled water for about 1 h to remove most of the ethidium bromide. The glyoxylated RNA was transferred to a presoaked (in 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) Genatran 45 filter (D and L Filter Corp.) as described by Thomas (34). After transfer, filters were baked in a vacuum oven for 2 h at 80°C.

For slot blots, phenol-extracted total RNA or RNA which had been separated into polysomal and nonpolysomal fractions was resuspended in distilled H_2O to a final concentration of 1 μg/ml. The RNA was denatured by the method of White and Bancroft (36). To 20 μl of RNA in a 1.5-ml tube was added 30 μl of TE buffer, 30 μl of 20× SSC, and 20 μl of 37% formaldehyde. The mixture was then incubated at 60°C for 15 min. Dilutions of the denatured RNA were made with 15× SSC. These were applied to a nitrocellulose filter with a Minifold II slot blotter (Schleicher & Schuell, Inc.) according to directions of the manufacturer. The actual amounts of RNA loaded in each dilution series were 10, 5, 2.5, 1.25, 0.6, and 0.3 μg. The filters were then baked as described above for the gel transfers.

Filter hybridizations. The cloned sequences used to probe for specific HSP RNA sequences have been described previously (15).

Northern filters or slot blot filters were prehybridized for 18 to 20 h at 42°C in a solution containing 50% formamide (Sigma Chemical Co.), 5× SSC, 1× Denhardt solution (0.02% each of bovine serum albumin, Ficoll [Pharmacia Fine Chemicals], and polyvinylpyrrolidone), 25 μg of yeast soluble RNA (type III; Sigma) per ml, 250 μg of denatured herring sperm DNA (Sigma) per ml, and 0.2% SDS. Labeled probes (ca. 10^6 cpm per filter) and dextran sulfate (Sigma; 10% final concentration) were added directly to the prehybridization mixture, and hybridization was carried out at 42°C for 12 to 20 h. The filters were washed and exposed to X-ray films (Kodak XS-5 or XAR-5) as described by Thomas (34).

Quantitation of autoradiograms. Appropriately exposed X-ray films (those in which the density of exposure was <2.5 absorbance units) were scanned on an LKB Ultrascan densitometer. Peak areas were determined by tracing the scans and then cutting out the peaks and weighing them.

RESULTS

When *T. thermophila* cells growing at 30°C were shifted to temperatures between 37 and 41.5°C, they showed an induction of HSP synthesis and, if maintained at those temperatures, survived at the 100% level over a 24-h period. A shift to any temperature higher than 41.5°C was lethal, the rate of killing being faster the higher the temperature (Fig. 1). As with other cells, a brief exposure to a nonlethal but HSP-inducing temperature allowed *Tetrahymena* cells to survive at lethal temperatures (Fig. 1 [see also Fig. 8]).

To determine which metabolic properties of cells failed first after a shift to a lethal temperature, we measured the amino acid-incorporating abilities of cells shifted from 30 to

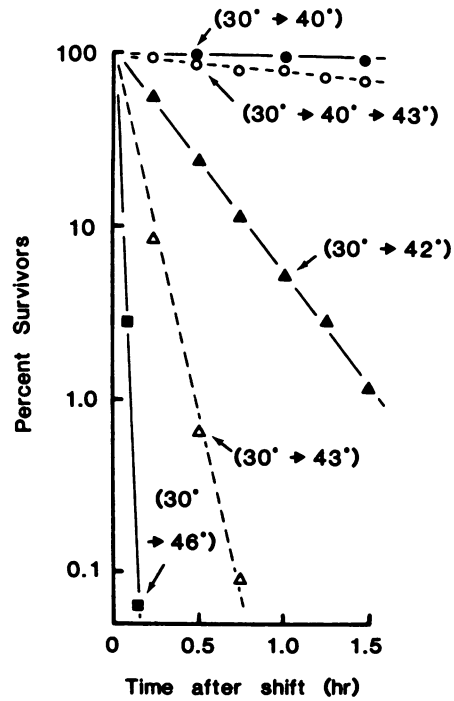

FIG. 1. Effects of various heat treatments on the viability of cells. In all experiments, the initial cell population to be tested was an early-log-phase culture growing at 30°C. Small volumes (about 5 ml) of cell cultures were transferred to prewarmed flasks in shaking water baths at the indicated temperatures and, at various times after the temperature shifts, aliquots of cells were removed and their viability was determined (see the text). The temperature shift is indicated for each survival curve. In one experiment, a culture was shifted from 30 to 40°C, allowed to remain at 40°C for 1 h, and then shifted to 43°C, after which viability measurements were made.

43°C and compared the results to those seen for cells shifted from 30 to 40°C. Both the quantitative (Fig. 2A) and the qualitative (Fig. 2B) aspects of this response were monitored. A shift to 43°C elicited an abrupt decline in amino acid-incorporating ability such that by 20 min after the shift the rate observed was <20% of the 40°C-treated control cells, and by 50 min the incorporation was indistinguishable from background labeling. The initial response of the cells shifted to 43°C was to begin synthesizing a typical array of HSPs, albeit at a reduced level. Subsequently, the rate of synthesis of these proteins declined rapidly. Also, in contrast to what was seen in the 40°C controls, no change in the relative synthesis rate of the different HSPs occurred.

Two possible explanations for these results were that insufficient levels of heat shock mRNAs were produced at 43°C or that some part of the translational machinery was functionally inactivated at this temperature. To distinguish between these two possibilities, the levels of hsp73 mRNA were measured for 1 h in cells shifted from 30 to 40°C and from 30 to 43°C. In both cases hsp73 mRNA levels increased in response to the elevated temperatures (Fig. 3a). Although the maximal increase in the 43°C-treated cells was somewhat less than that of the 40°C-treated cells at 30 min of heat shock, at 1 h of heat treatment the level of hsp73 mRNA in the 43°C-treated cells was identical to that found in the 40°C-treated cells. In a similar analysis using a cloned hsp80 gene (15) as a probe, the level of hsp80 mRNA showed identical changes in cells shifted to 40 and 43°C (data not shown). The level of rRNA in the 43°C-treated cells was also

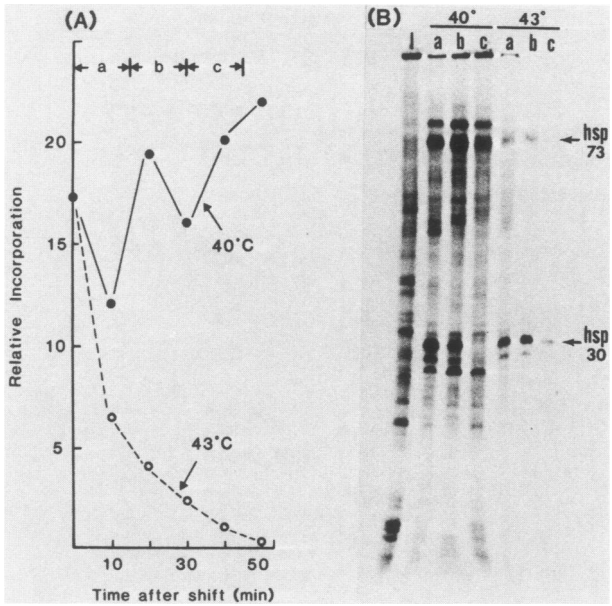

FIG. 2. Protein synthesis in cells heat shocked at 40 and 43°C. Early-log-phase cells growing at 30°C were transferred to either 40 or 43°C. (A) At various times 10-min pulses of [^{3}H]lysine were administered, and the overall incorporation rate of the cells was measured. (B) Cells were also labeled from 0 to 15, 15 to 30, and 30 to 45 min after having been shifted to 40 or 43°C, and their radioactive proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Lanes: 1, log cells at 30°C; a, b, and c, labeling periods at 40 or 43°C as indicated in panel A. The two HSPs indicated are really two (hsp73) and four (hsp30) different polypeptide species (Fig. 5c).

apparently unchanged after 1 h (Fig. 3b). Thus, unlike the situation in starved cells, in which a 40°C heat shock can sometimes cause rRNA degradation (15), ribosome destruction was not the cause of the decline in the protein synthesis rate. As expected, the vast majority (>95%) of the hsp73 mRNAs in the 40°C-treated cells at 30 min after the temperature shift were found to sediment with polysomes (Table 1). Surprisingly, the same percentage of hsp73 mRNAs in the 43°C-treated cells had identical sedimentation properties (Table 1). The fact that an initial synthesis of HSPs occurred after the temperature shift and that all of the hsp73 mRNAs were still loaded on polysomes after 1 h at 43°C indicated that the initiation of protein synthesis was not affected. Some other component(s) of the translational machinery must be altered by the elevated temperature in such a way as to inhibit elongation or termination and thus not allow for adequate synthesis of HSPs (or any other proteins, for that matter).

Cells heat shocked at 40°C for 30 min and then returned to 30°C cease detectable HSP synthesis within 10 min, and within 40 min they destroy at least 95% of the hsp73 and hsp80 mRNAs accumulated during the 40°C treatment (15; unpublished data). If after 1 h at 30°C these cells are returned to 43°C, they reaccumulate hsp73 and hsp80 messages with the same kinetics as those seen in Fig. 3 and now synthesize HSPs normally (data not shown). Thus, the prior 40°C heat shock produced a change in the translational machinery, rendering it functional at 43°C.

One candidate for modification during heat shock which could affect the translational properties of the cell is the ribosome (9, 31, 32). From our earlier work (14, 16) we knew that certain treatments (e.g., starvation in non-nutrient media and exposure to sublethal doses of a number of protein synthesis inhibitors) elicited changes in the structure of the ribosome. One treatment in particular, exposure to sublethal doses of cycloheximide, in addition to inducing ribosome structural changes, also appeared to alter the translational properties of the cell (14, 16, 35). To see whether sublethal heat shock treatment and cycloheximide treatment elicited a common functional response, we investigated whether a treatment which could induce a ribosome structural alteration could also induce thermotolerance to a 43°C treatment. Cells were treated at 30°C with a concentration of cycloheximide (0.5 μg/ml) to which they would adapt (8, 30); that is, protein synthesis and growth would be inhibited but the cells could recover and recommence growing. At various times during the adaptation, the protein synthetic activity of these cells at 30°C was monitored (Fig. 4a). At these times other cells were shifted directly to 43°C for 1 h, and their survival was monitored (Fig. 4b). Clearly, as the cells recovered the ability to synthesize proteins (i.e., as they adapted), a concomitant appearance of thermotolerance to a 1-h 43°C treatment (see also Fig. 8a) was observed. If no adaptation to the drug occurred (as seen in the cells treated with 5 μg of cycloheximide per ml), no acquired thermotolerance to 43°C was seen. This induction was not just an effect of the cycloheximide, since treatment with emetine, another drug to which *Tetrahymena* spp. can adapt (30), could also evoke the state of acquired thermotolerance (Table 2). Using a mutant strain with altered drug sensitivity, we found that the ability to acquire thermotolerance to 43°C required the transient inhibition of protein synthesis seen during the adaptation (Table 2).

An obvious question was then whether the adaptive response to the drugs elicited the synthesis of stress pro-

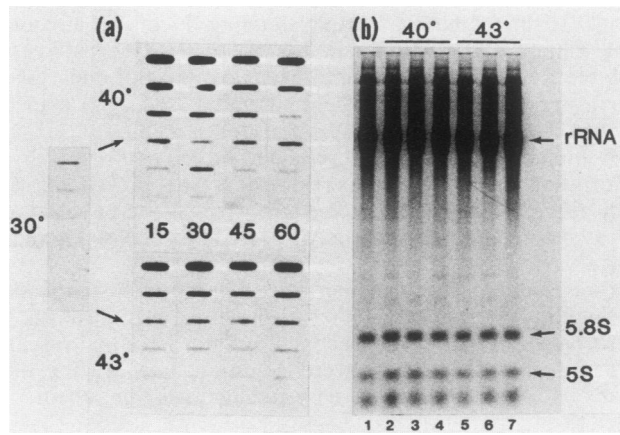

FIG. 3. Levels of specific RNAs in cells heat shocked at 40 and 43°C. Cells were treated as in Fig. 2. (a) At various times, total cellular RNAs were isolated, equivalent cell amounts (and dilutions thereof: 1/2, 1/4, 1/8, 1/16, and 1/32) were affixed to nitrocellulose filters, hybridized to a genomic clone of hsp73, and autoradiographed. RNAs were isolated at 15, 30, 45, and 60 min after the temperature shift. It should be noted that in the 40°C RNAs, two dilution series (30 and 60 min) have samples out of order. (b) Total cellular RNAs were also separated on 1.8% agarose gels, stained with ethidium bromide, and photographed. The RNAs were isolated from cells at 30°C (lane 1) or at 20 (lanes 2 and 5), 40 (lanes 3 and 6), or 60 (lanes 4 and 7) min after the temperature shift. The RNAs in lanes 2 to 4 were from cells at 40°C; those in lanes 5 to 7 were from cells at 43°C.

teins. To determine this, we pulse-labeled cells during adaptation to cycloheximide and analyzed the proteins synthesized at various times on one-dimensional (Fig. 5a) and two-dimensional (Fig. 5b) polyacrylamide gels. No obvious induction of the synthesis of the major HSPs was seen during the time when cells recovered from the effects of the cycloheximide inhibition. The same was true when cells adapted to a sublethal emetine treatment which induced thermotolerance to 43°C. During the period of time when cells rapidly became thermotolerant (90 to 150 min), a group of proteins was synthesized in a transient manner (Fig. 5a). This same array was apparent in emetine-treated cells as well (data not shown). However, none of these proteins was a major HSP (Fig. 5b).

To test further the idea that the recovery from protein synthesis inhibition was not eliciting a typical stress response, we measured the level of hsp73 mRNA in the cell during the period of drug adaptation and found no changes (Fig. 6). A similar analysis of hsp80 mRNA gave the same results (data not shown). However, at any time during the adaptation to cycloheximide at 30°C, cells were capable of responding to a 40°C (Fig. 6) or 43°C (data not shown) heat shock by increasing the levels of these messages to a normal, fully inducible level.

Although acquired thermotolerance to 43°C was induced during drug adaptation without the apparent synthesis of any HSPs, the following experiment showed that the reason drug-adapted cells could survive the 43°C treatment was that they were now capable of efficiently translating HSP mRNAs at 43°C. To measure the efficiency of HSP mRNA utilization in drug-adapted cells relative to nonadapted cells, we quantitated both the levels of hsp73 mRNAs and the protein synthesis capabilities of cells transferred from 30 to 43°C which either had or had not received a prior 3-h cycloheximide treatment. In both cases the temperature shift induced increases in the levels of hsp73 mRNAs to comparable levels (Fig. 7a). However, a normal HSP synthetic response was seen only in the cycloheximide-adapted cells while, as before, the nonadapted cells mounted an abortive response at 43°C (Fig. 7b).

That the synthesis of HSPs at 43°C in drug-adapted cells was absolutely required for their survival was demonstrated by the following experiment. If just prior to the shift to 43°C the cycloheximide concentration in the drug-adapted cell culture was raised to 15 μg/ml (a concentration which irreversibly inhibits amino acid incorporation to <2% of controls in less than 5 min), the cells died with the same kinetics as naive controls (Fig. 8a). This rate of killing

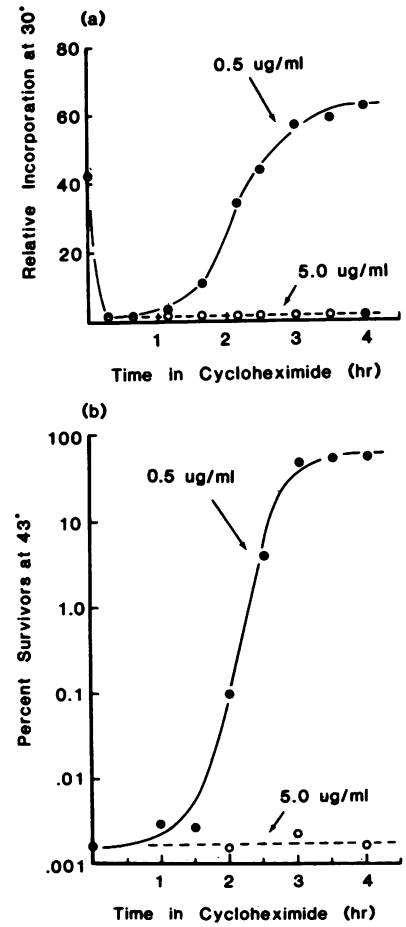

FIG. 4. Effects of cycloheximide treatment on protein synthesis and the ability to survive a 43°C heat shock. Early-log-phase cells at 30°C were exposed to cycloheximide at a concentration of either 0.5 or 5.0 μg/ml. At various times after administration of the drug, cells were either pulse-labeled at 30°C for 15 min with [^{3}H]lysine and their incorporation was measured (a) or a portion of the culture was transferred to 43°C for 1 h and the viability of the cells was determined (b). Whether cells were removed from cycloheximide-containing media or not before the 43°C temperature treatment had no effect on the results. Symbols: ●, cells treated with 0.5 μg of cycloheximide per ml; ○, cells treated with 5.0 μg of cycloheximide per ml.

TABLE 1. Distribution of hsp73 mRNAs in heat-shocked cells[a]

Temp (°C)	Fraction of total hsp73 mRNA in the cell			
	Untreated lysates		EDTA-treated lysates	
	Polysomal fraction	Postribosomal supernatant	Polysomal fraction	Postribosomal supernatant
40	0.98	0.02	0.06	0.94
43	0.95	0.05	0.08	0.92

[a] Early-log-phase cells growing at 30°C were shifted to elevated temperatures for 30 min. The postmitochondrial supernatants of lysed cells (containing >90% of all hsp73 mRNAs) were separated into polysomal and postribosomal (<80 S) fractions (see the text). Total RNA was isolated from each fraction, and the amounts of hsp73 mRNA in each fraction were determined by quantitating slot blots (Fig. 2). The fraction of the total hsp73 mRNA contained in the two compartments was then determined. EDTA release of mRNAs from polysomes was accomplished as described in the text.

contrasted with that seen when prior heat-shocked cells were treated in the same manner (Fig. 8a). These cells, in which HSPs had already accumulated, although unable to synthesize any more HSPs at 43°C (see the legend to Fig. 8 and Table 3), survived at a >50% level for 1 h and then showed a rapid decline in viability. This transient protection undoubtedly was due to the pre-accumulation by these cells of one or more of the HSPs. The fact that drug-adapted cells, in contrast to the prior heat-shocked cells, showed no ability to survive (above controls) at 43°C when inhibited from synthesizing proteins, confirmed our assertion that no HSP synthesis took place during the adaptation period at 30°C. It also showed that the HSP synthesis at 43°C which occurred in the adapted cells was both necessary and sufficient to insure their survival.

Finally, when cells, no matter what their prior physiological history, were shifted to 46°C, protein synthesis was completely abolished in less than 4 min (data not shown). A

TABLE 2. Effects of various concentrations of protein synthesis inhibitors on the induction of acquired thermotolerance in wild-type and cycloheximide-resistant cells[a]

Drug	Concn (μg/ml)	Wild-type cells		Cu-333 cells	
		Inhibitory effect on protein synthesis	Induction of acquired thermotolerance	Inhibitory effect on protein synthesis	Induction of acquired thermotolerance
Cycloheximide	0.5	Transient[b]	Yes	None[c]	No
	5	Total	No	Transient[d]	Yes/No[e]
	20	Total	No	Transient[b]	Yes
	40	Total	No	Total	No
Emetine	20	Transient[b]	Yes	Transient[b]	Yes

[a] Early-log-phase cells were exposed to drugs for 3.5 h. During this time, protein synthesis activity was monitored by pulse-labeling cells with [^{3}H]lysine. At the end of the drug treatments, cells were washed into fresh growth medium and exposed to 43°C for 1 h, and their survival was determined. Acquired thermotolerance was considered to have been induced if >50% of the cells survived the 43°C treatment. The cycloheximide-resistant strain (Cu-333) grows in concentrations of cycloheximide as high as 25 μg/ml (1).

[b] Protein synthesis was initially inhibited >90% by the drug treatment. By 3.5 h, full restoration of protein synthesis was observed.

[c] A slight initial inhibition (ca. 15%) was observed.

[d] A 60% inhibition was observed during the first 30 min, and by 1.5 h cells had returned to control levels.

[e] About 5% of the cells survived. This was considerably better than in the cases where no (<0.01%) survival was observed.

prior, nonlethal heat shock could prolong the viability of cells exposed to 46°C (Fig. 8b). However, although drug adaptation induced thermotolerance to 43°C as effectively as did a prior heat shock (Fig. 8a), it in no way endowed cells with an increased ability to survive at 46°C (Fig. 8b). The presence or absence of protein synthesis inhibitors in the cell at the time of shift to 46°C had no effect on the viability of cells (see legend to Fig. 8). Unlike the situation for cells transferred to 43°C, only if a prior accumulation of HSPs has occurred will cells be protected when shifted to 46°C.

DISCUSSION

Acquired thermotolerance is the phenomenon whereby a particular treatment of cells induces in those cells the ability

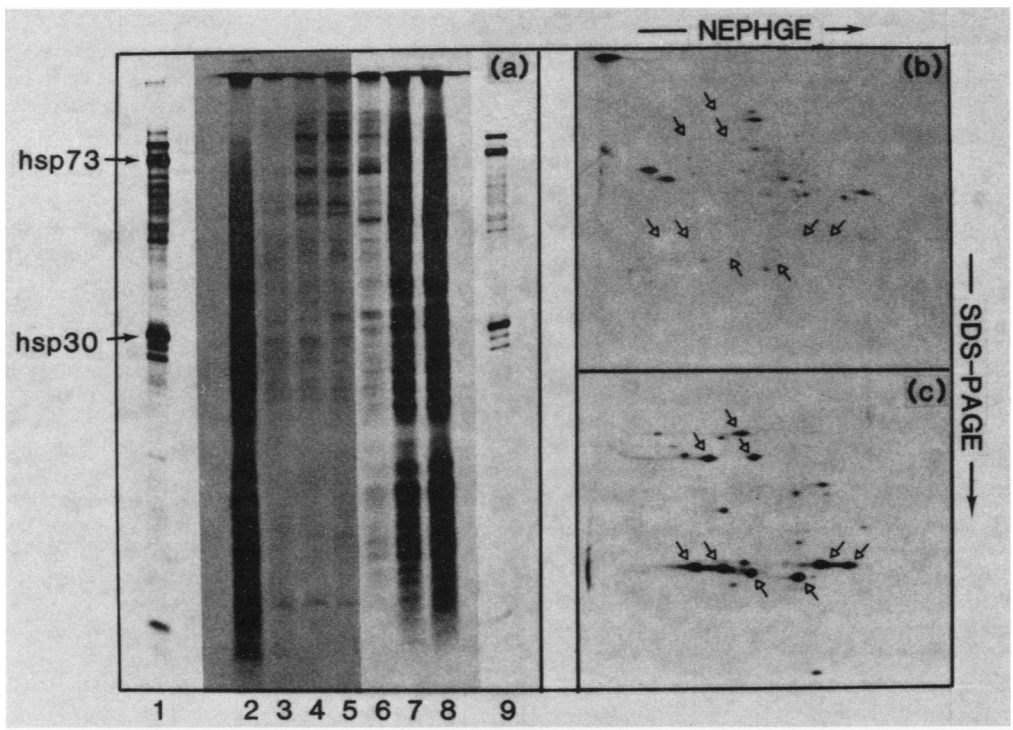

FIG. 5. Proteins synthesized by cells undergoing adaptation to cycloheximide. (a) Cells were treated with 0.5 μg of cycloheximide per ml as in Fig. 4, and the proteins synthesized in 20-min pulse-labelings with [^{3}H]lysine throughout the adaptation period (at 30°C) were separated on a 15% SDS-polyacrylamide electrophoresis gel and visualized by fluorography. Labeling periods: lanes 1 and 9, 5 to 35 min of a 40°C heat shock of non-drug-treated cells; lane 2, 20-min pulse of non-drug-treated log cells (at 30°C); lanes 3 to 8 (drug-treated cells), pulses of 30 to 50 , 60 to 80, 90 to 110, 120 to 140, 180 to 200, and 240 to 260 min, respectively. Since the various lanes contained markedly different levels of radioactive proteins, several autoradiographic exposures of the same gel were used to produce the photomontage in panel a. (b) Proteins synthesized by cycloheximide-treated cells labeled with [^{3}H]lysine at 30 to 120 min after drug administration were separated on a two-dimensional polyacrylamide gel and fluorographed. Proteins synthesized during the first 0.5 h of a normal heat shock and analyzed as in panel b are shown in panel c. The positions of the heat shock proteins in panel c are indicated in panel b by the arrows.

to better survive at what would otherwise be a lethal temperature. The results presented in this study suggest that two distinctions can be made. First, as a result of the way acquired thermotolerance is defined in an operational sense (i.e., whatever the particulars are of the heat treatment [temperature, duration of exposure] which cells are asked to survive), it may be that more than one path can be taken by those cells to achieve thermotolerance. Second, it is probably useful to distinguish between the ability to survive brief exposures to temperatures well above the minimum lethal temperature, the constant exposure to which a cell cannot survive, and the ability to survive for protracted periods at temperatures just one or two degrees greater than the minimum lethal temperature. This distinction is probably necessary since the mechanisms conferring resistance to killing in the former may not be totally congruent with those functioning in the latter. It is most likely that continued protein synthesis capacity is not required for short exposures to very high temperatures, whereas the ability to survive continuous hyperthermia requires a functioning protein synthesis machinery.

The data presented here indicate that the prior synthesis of hsp73 and hsp80 is not an absolute requirement for the ability of *T. thermophila* to survive after being shifted to 43°C, although once at 43°C HSP synthesis is required. This conclusion follows from an examination of the protein synthesis data (Fig. 5) and the mRNA analysis (Fig. 6) of the cells undergoing adaptation to cycloheximide. The evidence that synthesis of the other HSPs is not required in the induction of acquired thermotolerance to 43°C is less compelling. No obvious synthesis of any of the smaller-molecular-weight HSPs was observed at any time during adaptation to either cycloheximide or emetine. However, a low level of synthesis of one or more of these proteins throughout the adaptation period might have been missed. Until we have cloned copies of these other sequences we cannot determine whether any induction of these genes takes place during the adaptation period. In any case, no normal heat shock-like response, as is seen with agents which induce acquired thermotolerance in other organisms (21, 29), is elicited by either of these drugs. In another study (27), although cycloheximide treatment elicited the synthesis of a new set of proteins in *Neurospora crassa*, in agreement with what we report here, none of these appeared to be an HSP.

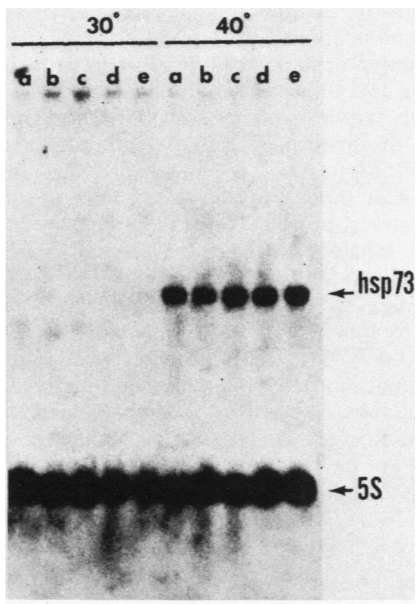

FIG. 6. Induction of hsp73 mRNA during cycloheximide adaptation. Cells in the early-log-phase of growth were treated with 0.5 μg of cycloheximide per ml at 30°C. At various times during adaptation to the drug, samples of the culture were transferred to 40°C for 30 min. Total cellular RNA was isolated from cells maintained at 30°C and from those shifted to 40°C, separated on 1% agarose gels, transferred to filters, and hybridized to nick-translated plasmids containing a genomic hsp73 sequence and a 5S rRNA sequence (15). Lanes: a, non-drug-treated cells; lanes b to e (drug-treated cells), pulses of 60, 90, 120, and 180 min, respectively. The 40°C heat treatment was administered during the last 30 min of each time point.

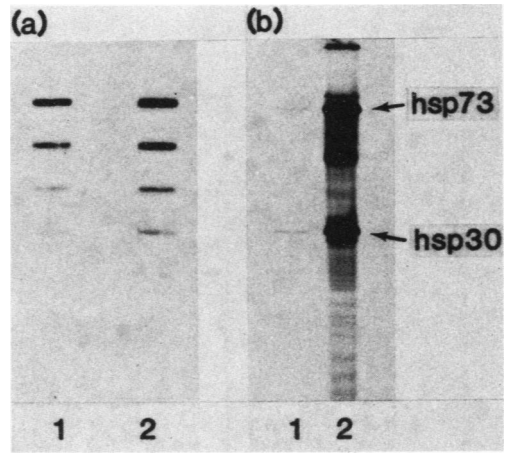

FIG. 7. Effects of cycloheximide adaptation on hsp73 mRNA metabolism and protein synthesis at 43°C. Early-log-phase cells were treated with cycloheximide (0.5 μg/ml) for 3 h at 30°C. These cells and non-drug-treated controls were then shifted to 43°C. At 30 min after the temperature shift, RNA was isolated from samples of these cells, and amounts (and sequential 1:2 dilutions) from equivalent cell numbers were affixed to membrane filters and hybridized to a labeled plasmid containing an hsp73 genomic insert (a). Lanes: 1, RNA from the non-drug-treated control; 2, RNA from the cycloheximide-adapted cells. Scans of the autoradiograms showed the drug-treated cells to have twice the amount of hsp73 mRNA per cell. Other samples of the drug-treated and control cells at 43°C were pulse-labeled with [^{3}H]lysine at 15 to 45 min after the temperature shift. Portions containing identical cell numbers and protein from each were separated by SDS-polyacrylamide gel electrophoresis (15% gel), and the radioactive proteins were visualized by fluorography (b). Lanes: 1, control; 2, cycloheximide-treated cells. The overall incorporation of lysine in the drug-treated cells was 20 times that of the nonadapted controls.

The simplest explanation for our results is that for cells to survive a brief 46°C heat treatment absolutely requires the prior accumulation of HSPs. However, for cells to survive an extended 43°C heat treatment requires the ability to synthesize HSPs throughout that period. Whether the HSPs are already present at the time of the temperature shift is not critical, although if already present they can confer temporary thermotolerance in the absence of additional HSP synthesis. Evidence that this second conclusion is probably correct comes from our preliminary analysis of a mutant strain isolated in the laboratory of P. Bruns (unpublished data). This strain, when heat shocked for 1 h at 40°C and then transferred to 43°C, showed a survival curve almost identical to that shown by wild-type cells which have been heat shocked at 40°C and then transferred to 43°C in the presence of high concentrations of cycloheximide (Fig. 8a);

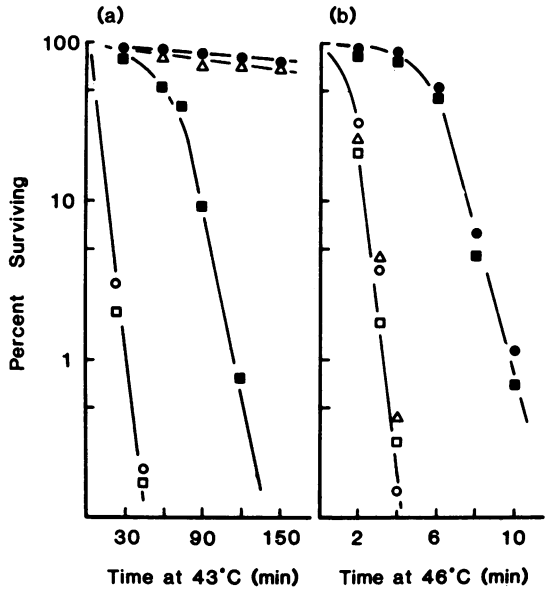

FIG. 8. Effects of various treatments on the viability of cells shifted from 30°C to either 43 or 46°C. The initial state of the cells to be tested was either early-log-phase growth at 30°C or just after a 3-h cycloheximide (0.5 μg/ml) treatment at 30°C. These cells were then transferred to either 40, 43, or 46°C. In the case of cells transferred to 40°C, after 1 h at that temperature they were subsequently transferred to either 43 or 46°C. In some experiments cycloheximide was added to a concentration of 15 μg/ml just before the transfer to a higher temperature. The viability of cells was measured beginning at the time of transfer to either 43 (a) or 46°C (b). The exact protocols followed and some characteristics of cells so treated are presented in Table 3.

that is, only a transient protection at 43°C was seen. This strain mounted a heat shock response at 40°C which was qualitatively and quantitatively indistinguishable (by two-dimensional gel analysis) from that elicited in a wild-type strain. However, when such cells were shifted to 43°C, further protein synthesis was inhibited, with kinetics identical to those seen in Fig. 2a. Cycloheximide and emetine treatment could not induce thermotolerance to 43°C in this strain. Thus, an apparently single mutation (R. L. Hallberg, preliminary data) can simultaneously eliminate the functional changes elicited by heat shock and by sublethal doses of protein synthesis inhibitors which would normally permit protein synthesis to occur at 43°C.

Even if HSP synthesis does not necessarily have to occur during the time a cell acquires the ability to withstand a subsequent shift to 43°C, our data do indicate that during this induction period, protein synthesis is required. This finding is at odds with the conclusion which Hall (13) reached with regard to the induction of acquired thermotolerance in the yeast strain *Saccharomyces cerevisiae*. Because exposing yeast cells to cycloheximide for 15 min at 23°C followed by an additional 60 min at 37°C induced acquired thermotolerance, Hall concluded that protein synthesis was not required for this induction. From our data, we suggest that perhaps during the 75-min exposure to cycloheximide the yeast cells could have recovered some protein synthesis capacity, thus allowing for the induction of acquired thermotolerance. This is not altogether unlikely as adaptation to cycloheximide inhibition in a manner not unlike that shown by *T. thermophila* has been reported in another yeast species (11). If true, it remains to be seen whether HSPs are made during the adaptive response.

What prevents naive cells from producing HSPs at 43°C? They can synthesize and accumulate HSP mRNAs at 43°C and even load them onto polysomes with apparently the same efficiency as cells which have an acquired thermotolerance, yet they quickly lose the ability to produce HSPs. These results suggest that either elongation (translocation) or termination steps (or both) of protein synthesis are in some way temperature sensitive above 41.5°C. This interpretation is not without precedence, since Ballinger and Pardue (2) have presented evidence suggesting that at normal heat shock-inducing temperatures in *Drosophila melanogaster*, non-heat shock messages may remain loaded on polysomes but are not translated. We observed that a similar phenomenon can apply to heat shock messages above a certain temperature. One explanation for our data is that, during drug adaptation (and a sublethal heat shock), structural modifications occur in either ribosomes or associated protein synthesis factors which permit proper elongation and termination at elevated temperatures. This assures the adequate synthesis of the stress proteins and allows for survival at these normally lethal temperatures. Whatever these modifications may be, however, although they are elicited by heat shock they do not require the synthesis of the major stress proteins and are therefore not directly affected by whatever role(s) the HSPs play. Ribosomal modifications elicited by heat shock which appear not to be directly related to HSP function and which can also be elicited by treatment with protein synthesis inhibitors have been observed (T. W. McMullin and R. L. Hallberg, manuscript in preparation). These modifications have the necessary requisite characteristics for their being involved in the translational changes we propose. Whether these modifications occur during drug adaptation is unknown but is currently being investigated.

TABLE 3. The protocols followed for the experiment described in Fig. 8 and some characteristics of the cells so treated

Treatment	Presence of HSPS in cells at time of last temp shift	Amino acid incorporation after temp shift	
		43°C	46°C
(○) 30 → 43 (or 46°C)	−	−	−
(●) 30 → 40 → 43 (or 46°C)	+	+	−
(△) Adapted → 43 (or 46°C)	−	+	−
(■) 30 → 40 → 43 (or 46°C) + cyc[a]	+	−	−
(□) Adapted → 43 (or 46°C) + cyc	−	−	−

[a] Abbreviation: cyc, cycloheximide.

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